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Identification of the Putative RNA Polymerase of *Cryphonectria* Hypovirus in a Solubilized Replication Complex

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Hypovirulence of the pathogenic fungus *Cryphonectria parasitica*, caused by the unencapsidated viral double-stranded RNA of *Cryphonectria* hypovirus (CHV1), provides a means for biological control of chestnut blight. We report here the isolation of a replication complex of the virus solubilized from host membranes. The conserved regions of the putative RNA polymerase encoded by strain CHV1-713 were cloned and expressed, and the recombinant protein was purified and used to produce polyclonal antibodies. The CHV1 replication complex was solubilized from a membrane fraction of CHV1-infected *C. parasitica* hyphae. Antibodies raised against the putative viral polymerase reacted on a Western immunoblot with an 87-kDa polypeptide of the replication complex but not with comparable preparations from an isogenic uninfected strain. Analysis of the polypeptide composition of the complex by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining revealed a number of other polypeptides along with the double-stranded RNA of the virus. We conclude that this 87-kDa polypeptide is the putative RNA polymerase encoded on open reading frame B of CHV1.

A new family of viruses, the *Hypoviridae*, has been discovered in the plant-pathogenic filamentous fungus *Cryphonectria parasitica*. The type species of this family of viruses, *Cryphonectria* hypovirus (CHV1, formerly hypovirulence-associated virus), has a double-stranded RNA (dsRNA) genome which is not infectious but can be transmitted vertically by fungal hyphal anastomosis and through asexual, but not sexual, spores (1, 14). In addition to being noninfectious, CHV1 differs from most other known viruses by the absence of a capsid (8). Replication of this virus is thus different from that of other dsRNA viruses in that most replicate in association with their capsids (12). CHV1 has received attention because of the unique phenotypic effect of the virus on its host: infected strains of the fungus sporulate poorly and have much lower than normal virulence. These effects of the virus on this plant pathogen are the basis for biological control of chestnut blight, the disease caused by *C. parasitica* (24).

The RNA polymerase activity of strain CHV1-713 was found to be associated with dsRNA-containing membrane vesicles isolated from an infected strain, but not those from a healthy strain, of the fungus (7, 8). The uninfected strain of the fungus contained a similar vesicle fraction, but these vesicles differed significantly in protein and lipid composition from those of the CHV1-infected strain (8). We have described the properties of the RNA polymerase associated with dsRNA-containing fungal vesicles and reported on the nature of the products synthesized in vitro (7). These RNA products hybridized specifically with CHV1 genomic dsRNAs. Hybridization to single-stranded cDNA clones showed that the reaction products were full-length copies of both strands of the dsRNA (7). Single-stranded RNA synthesis was found to be asymmetrical, with more than 80% of the polymerase products being of positive polarity. These results suggest that the RNA polymerase activity we detected was primarily transcriptase activity; however, some replicase activity (minus-strand synthesis) was detected in vitro (7). The in vitro reaction can thus account for all expected in vivo products of the replication cycle of this virus. In this report, we describe the isolation of a CHV1 replication complex from CHV1-infected hyphae.

Isolation and purification of CHV1 replication complex from fungal mycelia. The purification procedure used to isolate the CHV1 replication complex was modified to enable rapid handling of larger amounts of mycelia with a higher recovery of enzyme activity than the methods described previously (8). Cells were disrupted with a bead beater (Biospec Products, Bartlesville, Okla.), and the time for purification was reduced by eliminating the two overnight incubations in extraction buffer (6). Another change in the procedure was the use of the extraction buffer to resuspend all pellets until resuspension of the final high-speed centrifugation pellet in TMD buffer (50 mM Tris [pH 8.0], 10 mM $MgCl_2$, 5 mM dithiothreitol). These changes allowed isolation of the vesicle fraction (6, 8) in one working day. Vesicle preparations could be stored frozen at −70°C for more than 6 months in the presence of 15% glycerol without loss of polymerase activity. This fraction had 5% of the original activity, and there was a 277-fold purification of the polymerase, whose activity was assayed as previously described (7). Treatment of the vesicles with 0.5% Triton X-100 produced a soluble replication complex which was then further purified by centrifugation through a linear 5 to 20% (wt/wt, in TMD buffer) sucrose gradient for 4 h at 40,000 rpm and 4°C in a Beckman SW41 rotor. The peak fractions of polymerase activity were found near the top of the gradient (Fig. 1A). The fractions containing the most CHV1 dsRNA were those with the greatest polymerase activity (Fig. 1B). Under these centrifugation conditions, vesicles not treated with a detergent sedimented to the bottom of the tube, where the dsRNA and the polymerase activity were found. This purification step yielded 15% of the activity of the vesicle

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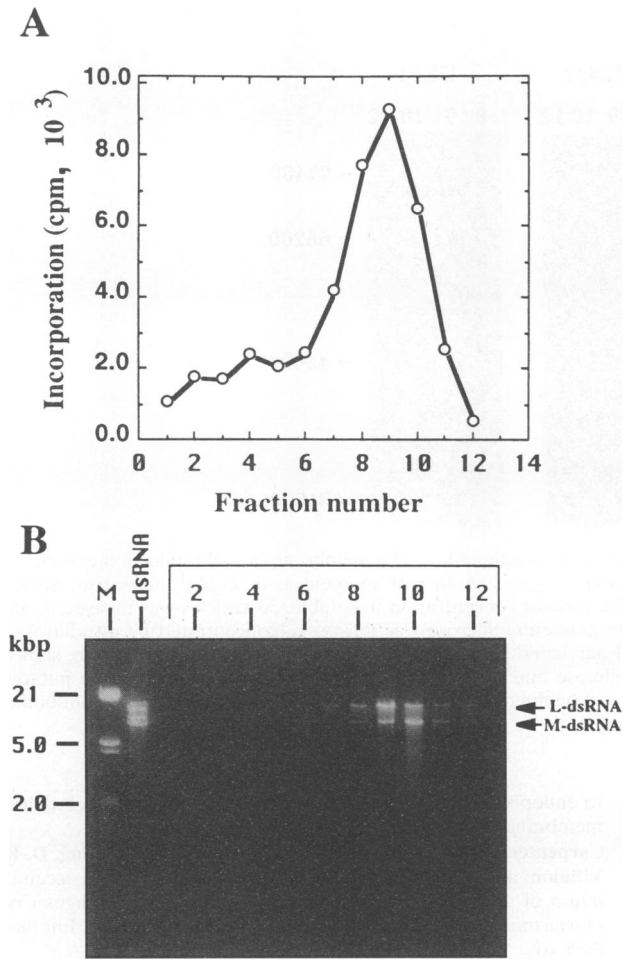

FIG. 1. Sucrose gradient analysis of replication complexes solubilized from fungal vesicles of a hypovirulent strain (UEP1) of *C. parasitica*. (A) Polymerase activity of each fraction (fraction 1 is the bottom fraction). A 25-μl aliquot of each fraction was mixed with an equal volume of twice-concentrated RNA polymerase reaction mixture. After 60 min of incubation at 30°C, 5 μl of the reaction mixture was spotted onto DE-81 filters to determine the amount of [^{32}P]UTP incorporated into RNA (20). (B) Agarose gel analysis of nucleic acids. Samples (30 μl) of the sucrose fractions were analyzed by electrophoresis in a Tris-borate-EDTA–1% agarose gel and visualized by ethidium bromide staining (lanes 1 to 12). The molecular size markers used were a *Eco*RI-*Hin*dIII digest of lambda DNA (lane M) and CHV1 dsRNA isolated and purified through CF11 cellulose column (lane dsRNA). L-dsRNA, 12.7-kb dsRNA; M-dsRNA, 8- to 10-kb dsRNA.

fraction with a 1.6-fold increase in specific activity. We do not know whether the soluble replication complex allows reinitiation, as does the vesicle fraction, or whether both strands of RNA are made by the soluble complex; the failure of these events to occur could explain the disproportionate loss of polymerase activity in this step. Products of the polymerase reaction yield labeled dsRNA of CHV1 when separated by agarose electrophoresis (data not shown).

Western immunoblot analysis of membrane-bound replication complex. PCR amplification was used to clone (5) the sequence from bp 7800 to 9029 of CHV1-713 (21), which corresponds to conserved domains of the putative RNA polymerase encoded in open reading frame B (ORFB) of the virus (13), into the vector pET3a (19). This clone (pPOL32) was expressed in *Escherichia coli* BL21(DE3)phLysE (23), and the purified protein (11) was used to make polyclonal antibodies against the putative RNA polymerase in rabbits. The antibodies were purified by using the expressed protein (22). The antibodies obtained in this way reacted specifically (9) with a single protein (87 kDa) present only in the solubilized complex isolated from the hypovirulent strain UEP1 (Fig. 2, fractions 9 and 10). This protein was present only in the fractions containing the peak RNA polymerase activity of the sucrose gradient (Fig. 1A, fractions 8 to 10). The 87-kDa protein did not react with preimmune serum, and it was not present in similar preparations of vesicles isolated from strain EP155/2, a CHV1-free strain isogenic to UEP1 (Fig. 2). This protein was also detected by the antibodies in a replication complex of CHV1 in the European strain EP113 (data not shown). This strain is the the source of CHV1 in UEP1 and EP713 and is not isogenic to EP155/2. These results suggest that the 87-kDa protein is the putative polymerase of CHV1. Identification of this protein, encoded by the viral polymerase sequences of ORFB, is the first evidence for *trans* processing of a polypeptide of ORFB of CHV1.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed that fractions of the sucrose gradient that contain the replication complex contained a number of other proteins (Fig. 2) which did not react with the antibodies. One protein which is near the size of the 87-kDa RNA polymerase was detected in each strain. The lack of reaction with antibody by the protein in EP155/2 shows these to be different proteins, although it does not eliminate the possibility that this other 87-kDa protein is also present in UEP1 along with the putative RNA polymerase. Some other proteins present in UEP1 were not detected in comparable preparations of the CHV1-free isogenic strain EP155/2 (Fig. 2). For example, the 60- and 66-kDa proteins, which can be clearly seen in fractions 8, 9, and 10 of UEP1 (Fig. 2A) were not detected in EP155/2 preparations (Fig. 2B).

The source of the membranes of the vesicle fraction from *C. parasitica* was not identified, and since the procedures used for their isolation are not typical of those used for isolation of cellular membranes, little can be concluded about their nature (3). The general paucity of proteins in the vesicle fractions isolated from both infected and uninfected strains of the fungus is atypical of most fungal cellular membranes (3). The yield of this fraction of membrane vesicles from virus-infected strains of the fungus is manyfold greater than that from uninfected strains, suggesting that the virus induces formation of this membrane fraction.

It is not clear whether any of the other proteins detected in the blots shown in Fig. 2 have any role in viral replication or whether their copurification with the polymerase activity and dsRNA (Fig. 1 and 2, fractions 8 to 10) is merely the result of their association with the vesicle fraction. Knowledge of the role of host proteins in RNA replication of plus-strand RNA viruses of eukaryotes is very limited, although association of host proteins with viral replication has been reported for several plus-strand RNA viruses (2, 10, 15, 16). A host protein was shown to be involved in replication of brome mosaic virus (BMV) (17). This host protein, which copurifies with the BMV RNA-dependent RNA polymerase and stimulates BMV minus-strand RNA synthesis in vitro, was identified as a subunit of eukaryotic translation initiation factor 3 (17).

An understanding of the molecular basis of transmissible hypovirulence in *C. parasitica* requires detailed knowledge of the structural and functional properties of hypovirulence-associated dsRNAs and their replication. We have reported

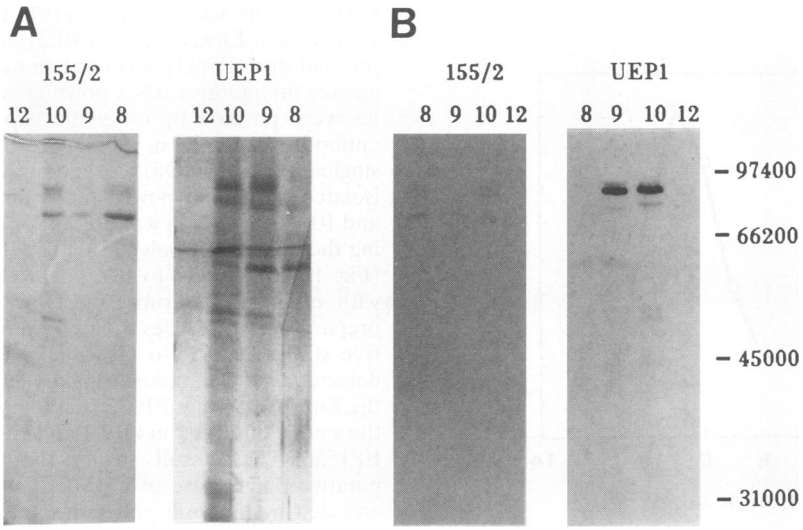

FIG. 2. Immunoblot analysis of replication complexes isolated by sucrose gradient fractionation. The membrane-bound replication complex of CHV1 from strain UEP1 was solubilized with Triton X-100 and separated along a 5 to 20% sucrose gradient as described in the text. Similar membrane fractions were prepared from the dsRNA-free strain EP155/2. The protein concentration in solubilized vesicles was measured, and equal amounts of protein of strains UEP1 and EP155/2 were loaded on the gradients. Sucrose fractions were concentrated by ultrafiltration (Centricon 10 microconcentrator; Amicon) and analyzed on an SDS–10% polyacrylamide gel. Only gradient fractions 8, 9, 10, and 12 are shown. (A) Silver-stained gel. (B) An identical gel was electroblotted onto nitrocellulose and probed with antibodies prepared against the putative recombinant polymerase expressed in *E. coli*. Molecular weight markers are shown on the right. A protein of 87 kDa is recognized by the antibodies in fractions containing the viral replication complexes (lanes 9 and 10 in panel B).

here that replication of these dsRNAs is associated with host membrane vesicles and that, despite induction of host membrane changes, replication of the virus does not appear to affect normal host growth. The symptoms of CHV1 infection in this host are limited primarily to perturbation of developmental events, such as sporulation, and virulence (4, 18, 25, 26). Since membrane vesicles are thought to play an important role in fungal growth, it is not clear how this virus can induce changes in host membranes without affecting growth of the fungus. An understanding of the processes, host structures, and molecules necessary for replication of CHV1 will be important in learning how this virus can replicate and perturb host development without affecting host growth. Isolation of a soluble complex with polymerase activity from these membranes and identification of the putative viral polymerase in this complex are significant initial steps in understanding the replication of this virus.

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